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GAMMA-AMINOBUTYRIC ACID UPTAKE AND METABOLISM IN APLYSIA DACTYLOMELA

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Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care," prepared by the National Academy of Sciences - National Research Council.

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ABSTRACT

Selected ganglia of Aplysia dactylomela were found to possess a sodium dependent gamma-aminobutyric acid (GABA) uptake system. Tissue to medium ratios as high as 20:1 were calculated in the buccal ganglion after a 45-minute incubation in 6 μM GABA. The metabolism of accumulated GABA was low with 90 percent of the radioactivity remaining as GABA after a 45-minute incubation and 20-minute washout period. The buccal ganglion displayed a sodium dependent, high affinity uptake system with a K_m = 52 μM. No net sodium dependent accumulation was found in the pedal ganglion. In the buccal ganglion, light microscope autoradiography indicated that the sites of GABA accumulation were predominantly extraneuronal.

I. INTRODUCTION

The Aplysia nervous system contains a number of identifiable nerve cells which have been used for neurochemical and electrophysiological investigations. Certain individual neurons have been shown to have cholinergic,[10,14,29] serotonergic[10,41] or adrenergic[1] properties (for review see Gerschenfeld[12]). Recently Brownstein et al.[5] observed that some of the identifiable Aplysia neurons contain more than one putative neurotransmitter. Zeman and Carpenter[43] found gamma-aminobutyric acid (GABA) present in greater than millimolar concentrations in several Aplysia neurons. Moreover, Gerschenfeld and Tauc[13] have found specific receptors in Aplysia ganglia which mediate hyperpolarizing or depolarizing responses to externally applied GABA.

Bennett et al.[2] suggested that the existence of a Na^+ dependent high affinity uptake system may be used as a criterion for putative neurotransmitters. Such a system could be significant in the inactivation of synaptically released GABA by effecting its removal from synaptic areas. Several investigators have shown that glial membranes may be the predominant sites of GABA uptake in invertebrates,[31] vertebrates[3,17,42] and cultured cells.[19,35] In other preparations, however, uptake of GABA into certain neurons has provided evidence consistent with its possible role as an inhibitory neurotransmitter (for review see Iversen[20]). Moreover, Morin and Atwood[30] reported that the amount of GABA taken up by a series of crustacean nerve-muscle preparations was dependent upon the degree of inhibitory innervation of each. Sisken and Roberts[38] found that GABA uptake in a crayfish stretch receptor was localized in regions innervated by inhibitory axons.

We present here the results of our studies on GABA uptake and metabolism in Aplysia ganglia, including the characterization of a Na^+ dependent, temperature sensitive, high affinity uptake system and the autoradiographic localization of the sites of uptake on the light microscope level. The results are interpreted with special emphasis as to the functional role of glia in GABA uptake.

II. MATERIALS AND METHODS

Aplysia dactylomela (200-400 g) were obtained from Marine Specimens Unlimited (Marathon, Florida) and kept in tanks of artificial seawater (17°C) for up to 2 weeks before use. Animals were pinned to dissecting trays and the ganglia with associated nerve trunks and connective tissue were removed. Individual ganglia were pinned to a paraffin block immersed in artificial seawater where nerve trunks and connective tissue were removed. The ganglion sac was then carefully lifted and slit, exposing the ganglion directly to the bathing medium. The ganglia were then transferred to separate flasks for incubations as described below. At the end of the incubations the ganglia were again pinned to a paraffin block so as to expose the opening in the sac. After blotting, the intact ganglion was removed from the sac and any adhering connective tissue.

In most experiments, one to four ganglia were immersed in 2 ml artificial seawater medium and were incubated in a shaking water bath at 20°C for 45 min. At the end of this time the medium was removed and the preparation was washed an additional 20 min in two changes of artificial seawater. The ganglia were then excised as described above and were homogenized in 0.05-0.6 ml of 0.1 N NaOH or 80 percent (v/v) ethanol.

The ganglia homogenized in ethanol were centrifuged at 5000 x g for 30 min at 0^{o}-5^{o}C to precipitate proteins. The pellets were washed twice with additional aliquots of 80 percent ethanol and were resuspended in 0.1 N NaOH for protein determination.[25] The combined supernatant fractions were collected in liquid scintillation vials containing Aquasol (New England Nuclear Corporation, Boston, Massachusetts) and were counted in a Nuclear-Chicago Mark II liquid scintillation spectrometer. Counting efficiencies were determined by the external standard ratio method. Portions of the NaOH homogenates were neutralized with HCl and were counted in the same manner.

The dependence of GABA uptake on Na^{+} was determined by incubating ganglia in media containing reduced amounts of Na^{+}. Temperature sensitivity was examined by incubating at 10^{o}C. To measure pH effects, incubation media were adjusted to pH = 3.5 prior to incubation. The effects of the pharmaceuticals chlorpromazine (10^{-4} M), bicuculline (10^{-4} M), and picrotoxin (10^{-4} M) on GABA uptake were surveyed by incubating the ganglia in the presence of these drugs and 6 μM ^{14}C-GABA. For all of these studies ganglia were preincubated for 10 min in media identical with the experimental media but lacking GABA.

Thin-layer chromatography of the ethanol extracts was done on 20 x 20 cm precoated glass plates (0.1 mm cellulose MN 300, Brinkmann Inst. Inc., Westbury, N. Y.). The solvent system most frequently employed was isopropanol-90 percent formic acid-H_2O (20:1:5). Radiochromatograms were cut into 1- to 5-mm strips which were removed from the glass plate with a razor blade and dispersed in Aquasol for scintillation counting.

For autoradiographic localization of the sites of GABA uptake, ganglia were incubated in artificial seawater containing 5 μCi/ml 2,3-^{3}H-GABA (10 Ci/mmole, 5 x 10^{-7} M) as described above. After the 20-min wash in two changes of artificial seawater containing no GABA, ganglia were fixed in a mixture of 1 percent glutaraldehyde and 4 percent paraformaldehyde[31] and postfixed in 2 percent OsO_4. This fixation procedure bound only about 30 percent of the ^{3}H-GABA accumulated by the ganglia. However, Orkand and Kravitz[31] found reasonable agreement in grain distribution between lobster tissues fixed in this fashion and similar freeze-dried tissues. Additionally, glutaraldehyde fixation has been successfully used by other investigators to demonstrate both neuronal[24] and glial[34] uptake of ^{3}H-GABA. After fixation ganglia were dehydrated in a graded ethanol series, vacuum embedded in Epon and sectioned at 1 μm. Sections were coated with Kodak NTB-2 Nuclear Emulsion by a roller method, exposed at 5^{o}C for 3-4 weeks, developed in Dektol (Kodak) and stained with toluidine blue.[40] Autoradiograms were photographed using a Zeiss Photomicroscope equipped with phase optics.

Artificial seawater was prepared from reagent grade salts in distilled water at the following final concentrations (meq/l): NaCl, 425; KCl, 10.1; $CaCl_2$, 20.5; $MgCl_2$, 44.1; $NaHCO_3$, 2.8; and $MgSO_4$, 52.4. Na^{+} free seawater was prepared by replacing the NaCl with equimolar quantities of Tris-Cl or LiCl or with half equimolar $MgCl_2$. (When $MgCl_2$ was used the medium osmolarity was maintained by adding sucrose.) The osmolarity of all media was 980-1000 mosmols and pH 7.8. U-^{14}C-GABA and ^{3}H-inulin were obtained from Amersham Searle Corporation (Arlington Heights, Illinois), 2,3-^{3}H-GABA was obtained from New England Nuclear Corporation

(Boston, Massachusetts) and 4-^{14}C-GABA from Mallinckrodt (St. Louis, Missouri). Radioisotopes were relyophilized before use but were not further purified provided the radiochemical purity remained above 97 percent as determined by thin-layer chromatography. Incubation media were prepared by suspending the relyophilized labelled GABA in artificial seawater containing, in some cases, unlabelled GABA or ^{3}H-inulin.

Extracellular space determinations were made using ganglia which were incubated in media containing ^{3}H-inulin or both ^{3}H-inulin and ^{14}C-GABA. Because over 60 percent of the total ganglion volumes were attributable to the extracellular space, data are expressed on a unit protein basis to provide a measure of the cellular material present. Measurements of the protein contents and volumes of identified Aplysia neurons[14, 28] indicate that these cells are approximately 10 percent (w/v) protein, i.e., 0.1 g protein/ml cellular volume. Therefore when the amount of radioactivity retained in the extracellular space has been subtracted, experimental data expressed as (dpm/0.1 g protein)/(dpm/ml medium) are approximately equal to the tissue:medium ratio (dpm/ml cellular volume)/(dpm/ml medium).

III. RESULTS

Survey of Aplysia ganglia. An initial survey of Aplysia ganglia was conducted to determine which were capable of accumulating ^{14}C-GABA from the external medium. The results of this survey (Table I) showed that each of the ganglia studied accumulated a greater amount of GABA in control media than in Na^{+}-free (Tris) media. The GABA accumulated in the absence of Na^{+} could possibly arise from (1) retention in the extracellular space, (2) passive intracellular diffusion, (3) incomplete removal of Na^{+} ions from the ganglia, or (4) a combination of these. The difference between the control

Table I. Accumulation of ^{14}C-GABA by Aplysia Ganglia*

Ganglia	Control	Na^+-free	Net
	(dpm/0.1 g protein)/(dpm/ml medium)		
Buccal	22.5 ± 1.9	2.5 ± 0.6	20.0
Pleural	15.3 ± 1.7	1.3 ± 0.2	14.0
Abdominal	9.3 ± 0.6	0.8 ± 0.1	8.5
Cerebral	8.5 ± 1.5	1.4 ± 0.2	7.1
Pedal	3.3 ± 0.4	1.6 ± 0.3	1.7

* Ganglia were incubated as described in Methods in artificial seawater containing 6 μM 4-^{14}C-GABA (2.01 mCi/mmole). Time course experiments showed uptake was linear at this concentration for the 45-min incubation period. In the Na^+-free medium equimolar amounts of Tris-Cl were substituted for NaCl. The accumulation measured in left and right members of symmetrically paired ganglia was similar. Each value is the mean ± S.E.M. for three to seven ganglia. Net accumulation is the difference between the accumulation in control and Na^+-free media and, on the basis of 10 percent (w/v) protein content, is equal to the tissue:medium ratio.

and Na^+-free data of Table I is taken as a measure of the net intracellular Na^+ dependent accumulation by each ganglion. This value was used to interpret the net Na^+ dependent accumulation data as ganglion tissue:medium ratios for the 45-min incubations in 6 μM GABA. Values ranged from 1.7:1 in the pedal ganglion to 20:1 in the buccal ganglion. Since these two ganglia had the highest and lowest net accumulation they were chosen for further study.

Metabolism of accumulated GABA. After ethanol extraction 99 percent of the radioactivity appeared in the combined supernatant fractions, and less than 1 percent in the protein pellet. Thin-layer chromatography of supernatant samples indicated

low catabolic rates for accumulated GABA. Ninety percent of the accumulated radioactivity had an R_f matching GABA standards. Based on these data the amounts of GABA accumulated in subsequent experiments were determined by measuring the total radioactivity in the ganglia.

Time course of GABA accumulation. The accumulation of GABA in the buccal and pedal ganglia was examined at various times for both low (5 μM) and high (1 mM) medium GABA concentrations (Figure 1). The results of these experiments showed

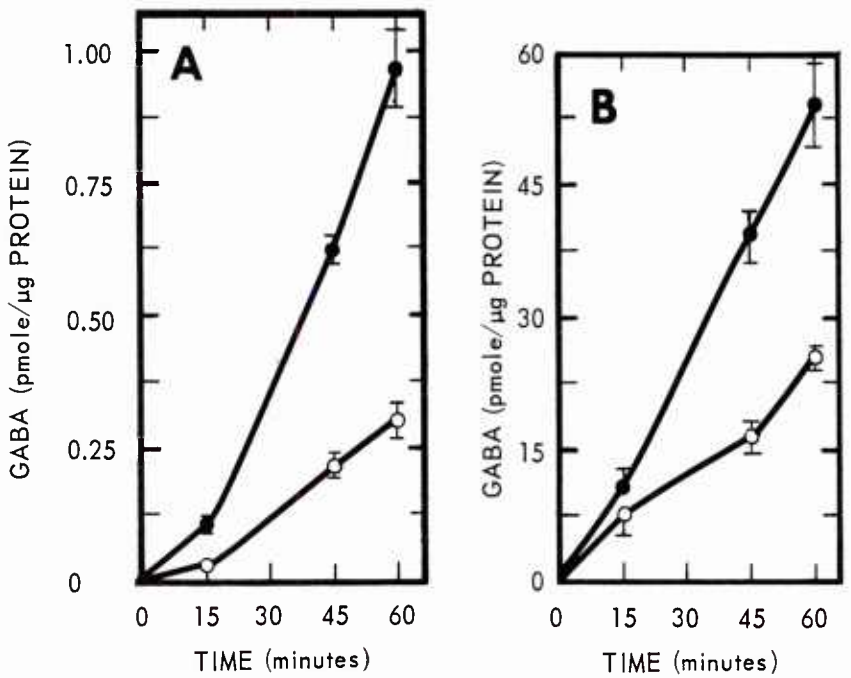

Figure 1. Time course of GABA accumulation. Incubations were conducted for various times in media containing either (A) 5 μM ^{14}C-GABA (228 mCi/mmole), or (B) 2,3-^{3}H-GABA (10 Ci/mmole, 0.1 μCi/ml) with 1 mM unlabelled GABA added. After each incubation ganglia were washed for 20 min in two changes of artificial seawater. The total amounts of GABA accumulated were determined from the specific activities of the media. Each point is the mean $\pm$ S.E.M. for three or four ganglia. (-•- buccal ganglion, -o- pedal ganglion).

that accumulation was linear throughout the 1st hour of incubation. At the low GABA concentration the initial rate of accumulation (0-15 min) appeared to be slower than at later times. This was probably due to progressive diffusion of the labelled GABA through the ganglia. Because uptake was linear at 45 minutes, this time was chosen for subsequent experiments.

Na^+ requirement of GABA accumulation. To ensure that the reduced accumulation of GABA observed in the Na^+-free medium was not due to some specific effect of the Tris, incubations were also conducted in media in which Na^+ was replaced by either Li^+ or Mg^{++} and sucrose (see Methods). The results of these incubations showed that GABA accumulation was no greater than in the media in which Na^+ had been replaced by Tris. Therefore it appears that the mechanisms responsible for GABA accumulation in Aplysia ganglia have an absolute Na^+ requirement.

In Figure 2 the dependence of GABA accumulation in the buccal ganglion on the medium Na^+ concentration is shown. In the Na^+-free media the amount of GABA accumulated was not significantly different from that expected to be retained in the extracellular space. At 1/6 Na^+ (71 mM) accumulation was about 60 percent control, and above 1/3 Na^+ (142 mM) no significant difference in accumulation from control media was observed. This nonlinear Na^+ dependence is similar to that observed by Iversen and Kravitz[21] for GABA uptake in a lobster nerve-muscle preparation and by Bennett et al.[2] for the uptake of other amino acids in rat brain synaptosomes. Detailed kinetic analyses performed by other investigators[26,27] have shown an apparent allosteric involvement of Na^+ in the transport of GABA by rat brain synaptosomes.

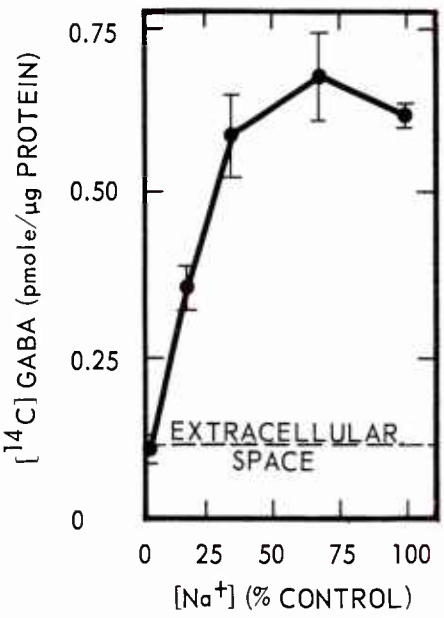

Figure 2.
Dependence of GABA accumulation on Na^+ concentration. Buccal ganglia were incubated as described in Methods in artificial seawater containing 5 μM ^{14}C-GABA (228 mCi/mmole) and varying concentrations of Na^+. The osmolarity of media deficient in Na^+ was maintained with Tris. Each point is the mean $\pm$ S.E.M. of three or four ganglia. The amount of GABA expected to be retained in the extracellular space is represented by the dashed line. This value was determined from the mean of six determinations of the amount of ^{3}H-inulin retained after similar incubations.

Effect of temperature, pH, and drugs. From Table II it can be seen that GABA uptake was decreased by temperature and low pH. The buccal ganglion was more sensitive to these effects than was the pedal ganglion. Chlorpromazine clearly decreased

Table II. Effect of Temperature, Low pH, and Drugs on GABA Uptake*

	Percent inhibition	
	Buccal	Pedal
10°C	32	21
pH 3.5	88	48
Chlorpromazine (10^{-4} M)	24	16
Picrotoxin (10^{-4} M)	<10	<10
Bicuculline (10^{-4} M)	--	<10

* Ganglia were incubated at the lowered temperature, pH or in the presence of chlorpromazine, picrotoxin, or bicuculline, and the effect of these conditions on uptake of ^{14}C-GABA (6 μM) was measured. Results are the averages of two ganglia and are expressed as percent inhibition relative to control.

uptake, however, the results from incubating with picrotoxin and bicuculline were either unclear or showed no significant effect.

The extracellular space. For the pedal ganglion the amount of ^{3}H-inulin present after a 45-min incubation (before washing) was 3.15 (dpm/0.1 g protein)/(dpm/ml medium) (Table III). On the basis of 10 percent (w/v) protein content (0.1 g protein/ml cellular volume, see Methods), this indicates that for every 1.0 ml of cellular volume there are 3.15 ml of extracellular space. The percentage of extracellular space in the pedal ganglion is then:

$$\text{percent extracellular space} = \frac{\text{extracellular volume}}{\text{extracellular + cellular volume}} \times 100$$

$$= \frac{3.15}{3.15 + 1.0} \times 100 = 76 \text{ percent.}$$

A similar calculation shows that the extracellular space accounts for 84 percent of the total volume of the buccal ganglion.

More GABA was accumulated in each ganglion than could be accounted for by the extracellular space alone (GABA/inulin > 1) both before and after the 20-min wash, indicating that the GABA was taken up intracellularly. In the buccal ganglion the 20-min wash removed 61 percent of the accumulated inulin but only 27 percent of the GABA, demonstrating that the GABA taken up was bound more tightly than was the inulin.

The fact that washing removed a smaller percentage of the inulin from the extracellular space of the pedal than of the buccal ganglion probably reflects the larger size of the former. However, the constant GABA-inulin ratio in the pedal ganglion before and after washing shows that even if the GABA was taken up intracellularly it was not tightly bound. Therefore it appears that passive diffusion may account for much of

the GABA taken up intracellularly in the pedal ganglion while an active uptake mechanism may be operating in the buccal ganglion.

Table III. Simultaneous Accumulation of ^{14}C-GABA and ^{3}H-Inulin*

	Pedal	Buccal
End of 45-min incubation		
Inulin	3.15	5.14
GABA	5.42	19.7
GABA/Inulin	1.7	3.8
After 20-min wash		
Inulin	2.12	2.03
GABA	3.96	14.4
GABA/Inulin	1.9	7.1
Percent washout		
Inulin	33	61
GABA	27	27

* Ganglia were incubated as described in Methods in artificial seawater containing 5 μM 4-^{14}C-GABA (2.01 mCi/mmole) and 0.05 μCi/ml ^{3}H-inulin (300 mCi/mmole). For each compound the data are expressed as (dpm/0.1 g protein)/(dpm/ml medium). Percent washout is the percent decrease in activity after the 20 min wash in artificial seawater containing no GABA or inulin. Each datum is the mean of two determinations which differed by an average of 8 percent from the mean.

Kinetics of the Na^{+} dependent uptake. To determine the dependence of GABA accumulation on media GABA concentration, ganglia were incubated in media containing ^{3}H-GABA and from 5 μM to 1 mM unlabelled GABA. Figure 3 shows that from

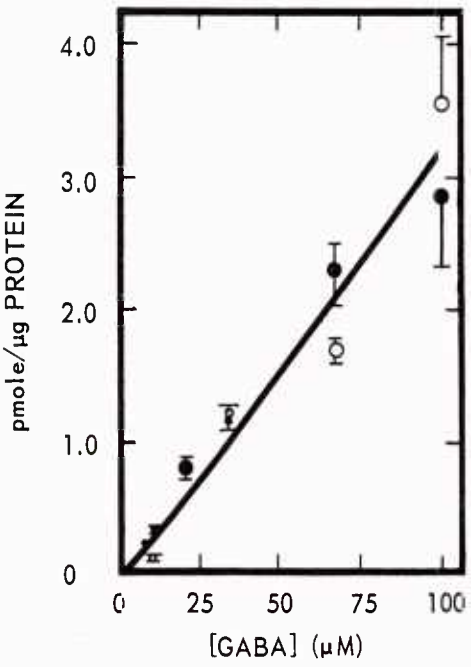

Figure 3.
GABA accumulation in the pedal ganglion. Ganglia were incubated as described in Methods in control (-•-) or Na^+-free (Tris) media (-o-) containing ^3H-GABA (0.2 µCi/ml, 10 Ci/mmole) and unlabelled GABA (5-100 µM). The total amounts of GABA accumulated were calculated from the net specific activities of the media. Each point is the mean ± S.E.M. for three to five ganglia.

5-100 µM GABA there was no significant net Na^+ dependent accumulation of GABA in the pedal ganglion. In the buccal ganglion (Figure 4) more GABA was accumulated at each concentration in control media than in the Na^+-free media.

The linearity of GABA accumulation at low concentrations in the Na^+-free media indicates that this accumulation was due to either retention in the extracellular space or passive intracellular diffusion. In previous experiments we found that the amount of GABA accumulated in Na^+-free media could be accounted for completely by retention in the extracellular space, both at low concentrations (5 µM, see Figure 2) and high concentrations (1 mM, data not shown). The extracellular space measurements also indicated that some shrinkage of the ganglion occurs in media containing 1 mM GABA. This phenomenon may explain the nonlinearity of the Na^+-free accumulation at higher concentrations. Additionally, in experiments conducted using control media containing

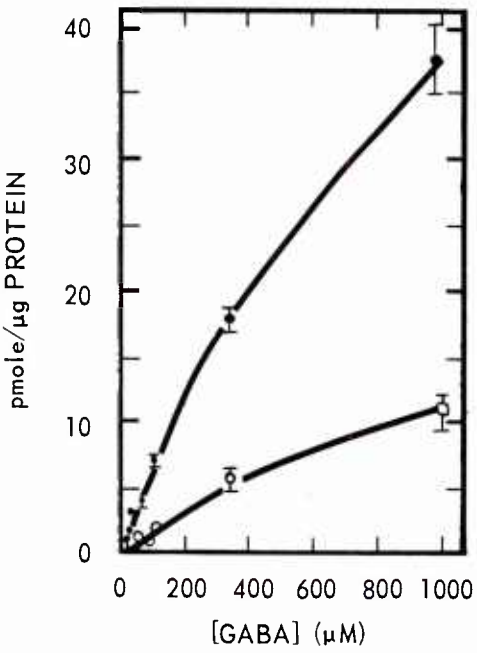

Figure 4.
GABA accumulation in the buccal ganglion. Ganglia were incubated in control (-•-) or Na^+-free (-o-) media as described for Figure 3. Net GABA accumulation in the Na^+-free media appeared to be linear for medium GABA concentrations up to 333 μM.

2 mM GABA, the total accumulation of GABA was significantly less than at 1 mM. This may in part have been due to saturation of an active uptake mechanism. However, the possibility of either shrinkage or deterioration of the ganglion precludes accurate interpretation of the data at these high GABA concentrations.

A Lineweaver-Burke plot of the net Na^+ dependent (control minus Na^+-free) component of GABA accumulation in the buccal ganglion is shown in Figure 5. The data appear to lie in two linear regions, over the concentration ranges 5-66.7 μM and 100 μM-1 mM. Linear regression analysis of the data in each concentration range gives apparent K_m values of 52 μM and 0.73 mM. As indicated above, little significance can be attributed to the higher K_m value. However, the lower value is taken to represent the apparent affinity constant of a high affinity Na^+ dependent saturable uptake system. A similar K_m was found by Orkand and Kravitz[31] for GABA uptake in a lobster nerve-muscle preparation.

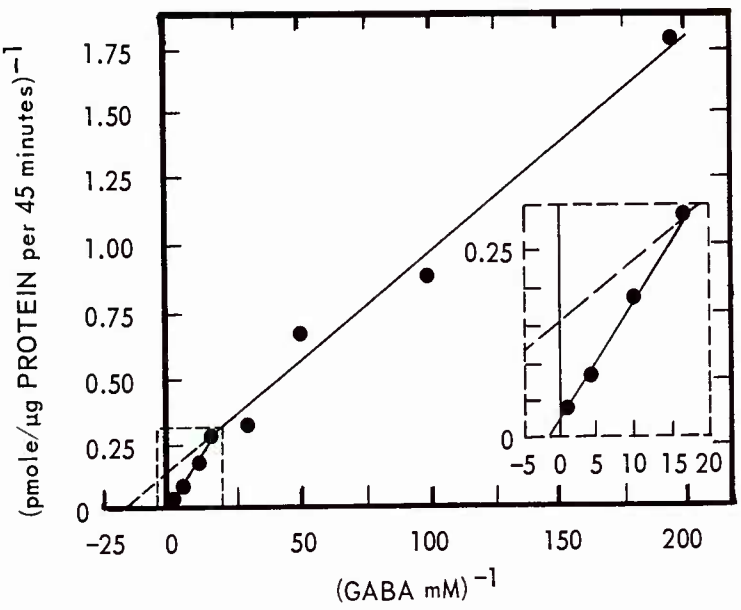

Figure 5. Lineweaver-Burke plot of the Na^+ dependent component of GABA uptake in the buccal ganglion. Each point is the difference between mean GABA accumulation in control and Na^+-free media for the data presented in Figure 4. Na^+-free data were not obtained at 10 or 20 μM so the predicted values from least squares analysis of the other data were used (see Figure 4). The two straight lines were obtained by least squares analysis of the data in the concentration ranges 5-66.7 μM (five points) and 0.1-1 mM (three points).

Localization of the sites of GABA uptake. Figures 6-9 are light microscope autoradiograms of sections of Aplysia buccal and abdominal ganglia. Glutaraldehyde-paraformaldehyde fixed ganglia were prepared as described in Methods after a 45-min incubation in 0.5 μM 2,3-^{3}H-GABA. Bar length equals 20 μm in all micrographs. These figures show that the sites of GABA uptake were almost exclusively extraneuronal. Labelling was concentrated around the periphery of nerve cell bodies and in the neuropil. Neuronal nuclei appeared devoid of silver grains. Some areas of neuronal cytoplasm contained grains, but these were usually associated with regions

Figure 6. Longitudinal section of a nerve trunk emanating from the buccal ganglion. Few silver grains occur within individual nerve fibers (Nf). Most grains are associated with the more heavily stained extraneuronal structures and structures around the periphery of nerve fibers (starred arrows). Some grains are also found in the connective sac (S). X 766.

invaginated by glial cells (Coggeshall[6] gives a complete description of the pronounced glial investment associated with Aplysia neuronal soma). Although nerve trunks emanating from the ganglia were heavily labelled, we were unable to identify any individual nerve fibers with high grain density. Projections of connective tissue into the ganglia were only sparsely labelled, as were the outer portions of the connective sac. Labelling did occur in inner regions of the connective sac immediately adjacent

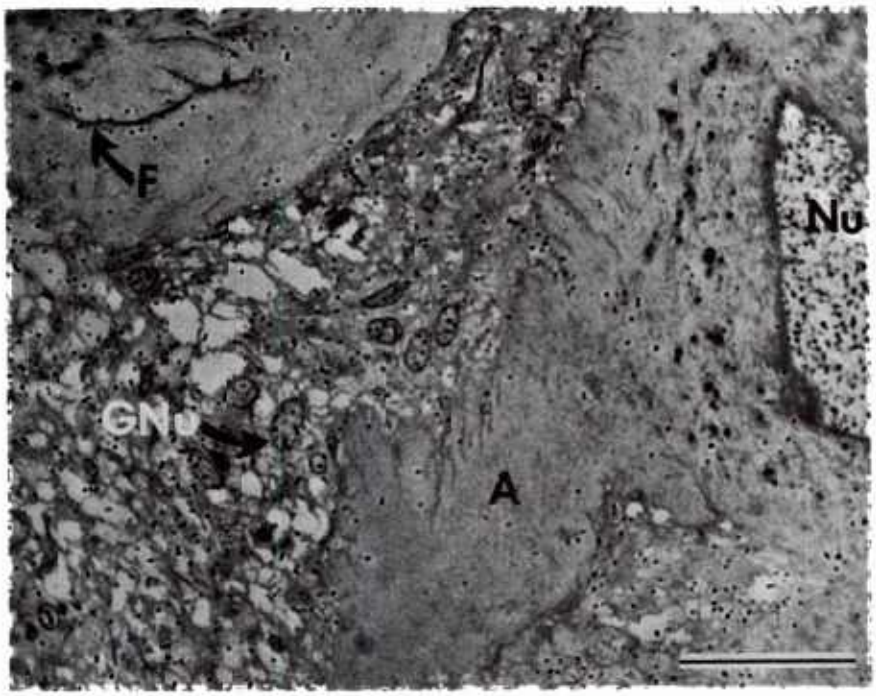

Figure 7. Two large buccal ganglion neuronal cell bodies. Silver grains are located predominantly over extraneuronal structures; however, few grains appear within glial cell nuclei (GNu). The neuronal nucleus (Nu) is devoid of grains as is the axon hillock (A) region of the large neuron. The nucleus and cytoplasm contain many pigment granules which could be confused with grains but are of a larger size and in a different plane of focus. Several grains appear in the neuronal cytoplasm, and appear to be associated with a glial fold (F). X 1032.

to the ganglia. These regions contained what appeared to be glial cells. The possibility of error in localization due to redistribution of label during fixation and embedding is unlikely, based upon very low grain density over extracellular space. Almost all the grains were associated with structure. As seen in the figures, pigment

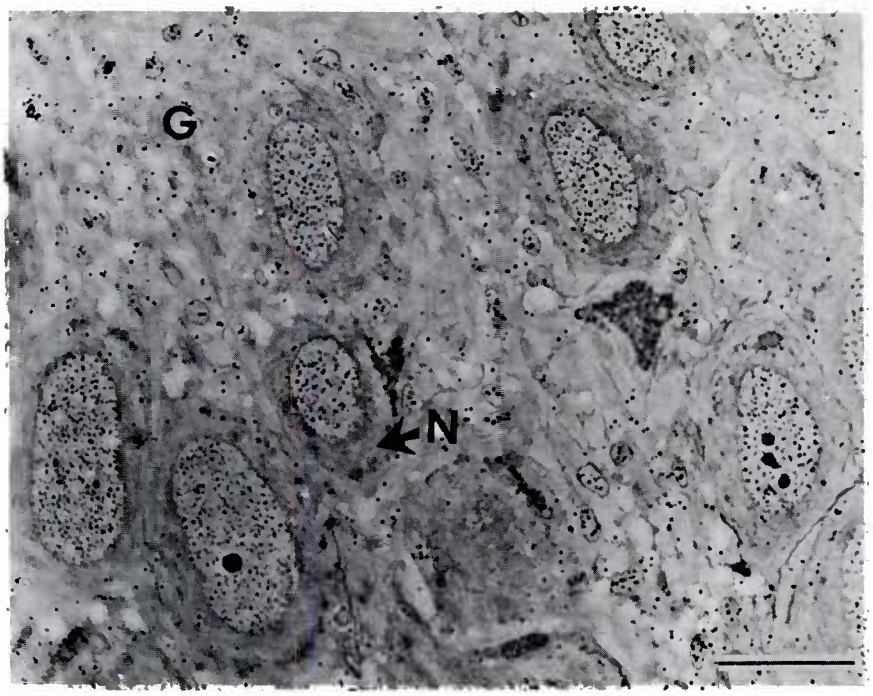

Figure 8. Several small neuronal cell bodies within the buccal ganglion. Few grains are associated with the small neuronal cell bodies (N) found by Zeman and Carpenter[43] to contain GABA in concentrations higher than several other identified Aplysia neurons. The nuclei of neurons contain many pigment granules as noted in Figure 7. Silver grains lie predominantly over extraneuronal, presumably glial (G), structures. X 825.

granules are present in the nuclei and cytoplasm of many neurons. These were distinguished from autoradiographic grains because of their size, color (pigment granules stained intensely blue) and also because they were in a different plane of focus than the silver grains.

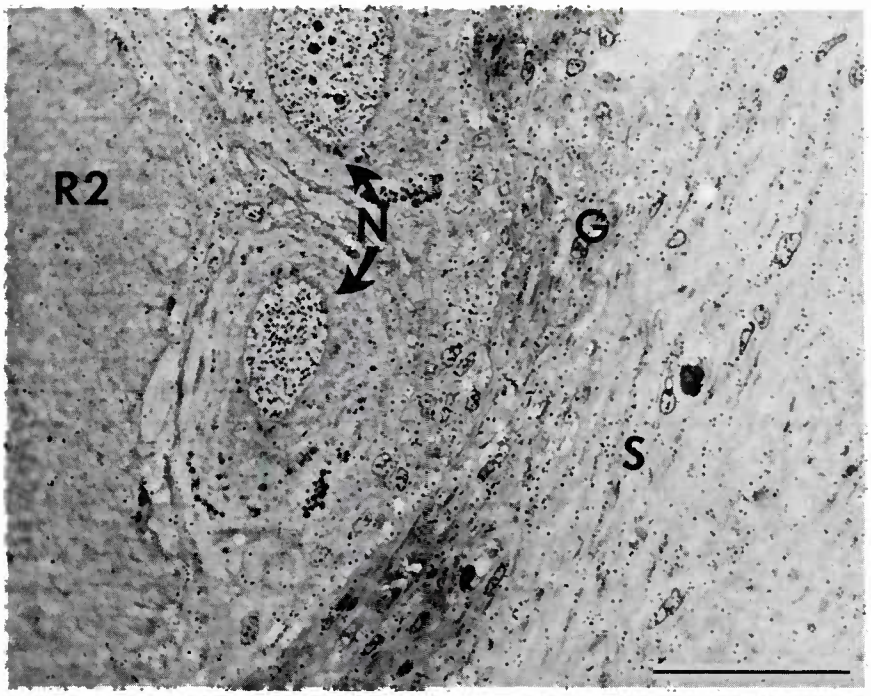

Figure 9. Portion of the Aplysia abdominal ganglion. The cytoplasms of the glial cell (R2) and other identified and unidentified neuronal cell bodies (N) of the abdominal ganglion were only sparsely labelled as compared to extracellular structures. The nuclei and cytoplasm of neurons contain pigment granules not to be confused with autoradiographic grains. The connective tissue of the ganglion sac (S) contained some silver grains but was more heavily labelled in regions adjacent to the ganglion containing glial cells (G). X 1230

IV. DISCUSSION

The finding that a high affinity sodium dependent uptake system for GABA exists in the buccal, but not in the pedal ganglion, demonstrates anatomical specificity for this amino acid in the Aplysia nervous system. Autoradiographic results further indicate that GABA uptake is most likely associated with glia in the buccal ganglion. This observation, along with data suggesting low activities of glutamate decarboxylase

for GABA synthesis,[12] raises the question as to whether GABA functions as a neurotransmitter in this system. Neuronal uptake of GABA has been demonstrated in vertebrate nervous tissues, supporting the view that GABA uptake is associated with rapid transport systems in those neurons which release GABA at their synapses.[9,15,18,23,24,39] If neurotransmitter uptake is strictly associated with the neurons that release the neurotransmitter, then the results presented here could argue against a transmitter role for GABA in Aplysia. However, the possibility exists that the high affinity GABA uptake system studied here is glial and functions to remove synaptically released GABA in order to terminate synaptic activity. Henn et al.[16] have suggested such a role for glutamate uptake by glia. In contrast to this, Young et al.[42] have suggested that glial uptake of GABA may serve only to protect neuronal membranes from excessive extracellular GABA. We feel that the evidence presented in this study, along with several other lines of evidence, argues for a unique role of glial uptake. Low turnover rates for GABA, an apparent glial specificity for GABA uptake, the intimate anatomical association of glial and nervous tissue, and the possibility of transport of GABA taken up by glia to neurons, strongly suggest that glia may play a critical role in neurotransmitter function and maintenance of GABA levels in the neuron.

The results of this study show that accumulated GABA has a low turnover rate. Others have indicated equally low[32] or nonexistent[4,33] rates of formation of GABA from its precursor, L-glutamic acid, via L-glutamic acid decarboxylase in molluscs. If the neurons are not synthesizing GABA very rapidly, then it would seem reasonable that some other means should exist for replenishing released GABA if the neurons that

release it do not have a mechanism for reuptake. One possibility that has remained unexplored in molluscs is the enzymatic formation of GABA from putrescine. Although Cottrell[7] was unable to detect putrescine in Aplysia, Dolezalova et al.[8] found that this four carbon diamine exists in measurable quantities in molluscan nervous tissue. Incorporation of radioactivity into GABA from labelled putrescine has been shown to occur in the brains of fish[36] and rats.[37] This pathway may be of some significance in maintaining endogenous GABA levels in nervous systems with little glutamic acid decarboxylase activity.

From the data presented here, it is clear that there exists some degree of glial specificity with regard to GABA uptake. However, the autoradiographic results in this study do not definitely rule out the possibility of some GABA uptake in neurons. Glia present in the pedal ganglia fail to show a sodium dependent, high affinity uptake mechanism. Alternatively, Schon and Kelly[34] have suggested that all glial cells have the capacity to accumulate exogenous GABA and that this function may only play a physiological role in regions where GABA is a neurotransmitter. Thus our data seem to indicate that there may be unique populations of glia capable of accumulating exogenous GABA. The range of tissue:medium ratios found in Aplysia ganglia (Table I) may represent different proportions of glia present in each ganglion which either possess or lack the ability to accumulate GABA. This hypothesis would be consistent with the suggestions of Schrier and Thompson,[35] Henn and Hamberger[17] and Hutchison et al.[19] that glia exert functional control over extracellular GABA concentrations in addition to inactivating synaptically released GABA. A similar function was suggested by Orkand and Kravitz[31] in the lobster.

The possibility of transport of accumulated GABA by glia to neurons is inferred from the neurochemical and anatomical data obtained in this study. The extensive glial association with neuronal perikarya, especially in the region of the axon hillock, has been generally assumed to be related in some way to the nutritive needs of the neurons.[6] Our observations that catabolic rates for accumulated GABA were low and that silver grains appeared in regions of neuronal cytoplasm containing glial indentations are consistent with this hypothesis. Lasek et al.[22] have recently demonstrated transport of Schwann cell proteins into squid giant axon, suggesting that mechanisms for transport of material from glia to neurons do exist.

In addition to the more physiological, high affinity, uptake system for GABA, there exists a separate low affinity GABA accumulation at high exogenous GABA concentrations. Using high GABA concentrations, Schrier and Thompson[35] and Hutchison et al.[19] found that GABA was accumulated by glia by what appeared to be diffusion. Because of the apparent shrinkage or deterioration we observed in Aplysia ganglia at these nonphysiologic exogenous GABA concentrations (>0.3 mM), our data in this range must be interpreted with caution. The observation that at high concentrations GABA accumulation was mediated by a low affinity uptake mechanism (K_m = 0.73 mM) is in agreement however with the finding of Evans[11] that glutamate uptake in crab peripheral nerve is mediated by a sodium dependent mechanism with the K_m = 0.28 mM. If intracellular diffusion of GABA does occur in Aplysia ganglia it would seem also to be a sodium dependent process since the amounts of GABA accumulated in the absence of exogenous sodium could be accounted for by retention in the extracellular space.

Early reports of the low GABA content of molluscan nervous tissue relative to other species, plus the apparent lack of measurable glutamic acid decarboxylase activity, have for some time discouraged the acceptance of a possible neurotransmitter role for this amino acid in molluscs.[12] The existence of specific receptors for GABA in Aplysia ganglia (Yarowsky and Carpenter, unpublished observation) and the recent demonstration of high (>1 mM) GABA concentrations in individual Aplysia neuronal cell bodies[43] have necessitated reevaluation of this position. The presence of a high affinity sodium dependent uptake mechanism for GABA, albeit glial, in at least one but not another ganglion demonstrates a marked degree of anatomical specificity for this amino acid in the Aplysia nervous system. In addition, we feel there is a definite possibility that GABA taken up into glial tissue is reutilized by transport to neurons which in turn release the GABA at the synapse. It is clear that further investigations as to the role of glia and glial specificity in relation to the neurotransmitter GABA are needed. The synthesis, release, sites of action, ultimate degradation, and transport of GABA require further study to evaluate the neurotransmitter role of GABA in Aplysia.

V. SUMMARY

Selected Aplysia ganglia accumulated exogenous GABA to a greater extent in the presence than in the absence of sodium. In the buccal ganglion a tissue to medium ratio of 20:1 was obtained for sodium dependent accumulation after a 45-min incubation in 6 μM ^{14}C-GABA. In the absence of external sodium the amount of GABA accumulated could be accounted for by retention in the extracellular space. In the pedal

ganglion no significant sodium dependent accumulation occurred for exogenous GABA concentrations from 5 to 100 μM.

The sodium dependent accumulation of GABA in the buccal ganglion was mediated by a high affinity component (K_m = 52 μM) and by either a low affinity component (K_m = 0.73 mM) or diffusion. Light microscope autoradiography showed that the sites of GABA accumulation in the buccal ganglion were predominantly extraneuronal. These results demonstrate anatomical specificity for GABA in the Aplysia nervous system, and indicate specificity among glial cells as to the capacity to accumulate exogenous GABA. This mechanism may be significant in controlling extracellular GABA concentrations, in inactivating synaptically released GABA, or in maintaining GABA available to the neurons.

REFERENCES

1. Ascher, P. Inhibitory and excitatory effects of dopamine on Aplysia neurones. J. Physiol. 225:173-209, 1972.

2. Bennett, J. P., Jr., Logan, W. J. and Snyder, S. H. Amino acids as central nervous transmitters: the influence of ions, amino acid analogues, and ontogeny on transport systems for L-glutamic and L-aspartic acids and glycine into central nervous synaptosomes of the rat. J. Neurochem. 21:1533-1550, 1973.

3. Bowery, N. G. and Brown, D. A. γ-Aminobutyric acid uptake by sympathetic ganglia. Nature (New Biol.) 238:89-91, 1972.

4. Bradford, H. F., Chain, E. B., Cory, H. T. and Rose, S. P. R. Glucose and amino acid metabolism in some invertebrate nervous systems. J. Neurochem. 16:969-978, 1969.

5. Brownstein, M. J., Saavedra, J. M., Axelrod, J., Zeman, G. H. and Carpenter, D. O. Coexistence of several putative neurotransmitters in single identified neurons of Aplysia. Proc. Natl. Acad. Sci. USA 71:4662-4665, 1974.

6. Coggeshall, R. E. A light and electron microscope study of the abdominal ganglion of Aplysia californica. J. Neurophysiol. 30:1263-1287, 1967.

7. Cottrell, G. A. Serotonin and free amino acid analysis of ganglia and isolated neurones of Aplysia dactylomela. J. Neurochem. 22:557-559, 1974.

8. Dolezalova, H., Giacobini, E. and Stepita-Klauco, M. An attempt to identify putative neurotransmitter molecules in the central nervous system of the snail. Int. J. Neurosci. 5:53-59, 1973.

9. Ehinger, B. and Falck, B. Autoradiography of some suspected neurotransmitter substances: GABA, glycine, glutamic acid, histamine, dopamine, and L-dopa. Brain Res. 33:157-172, 1971.

10. Eisenstadt, M., Goldman, J. E., Kandel, E. R., Koike, H., Koester, J. and Schwartz, J. H. Intrasomatic injection of radioactive precursors for studying transmitter synthesis in identified neurons of Aplysia californica. Proc. Natl. Acad. Sci. USA 70:3371-3375, 1973.

11. Evans, P. D. The uptake of L-glutamate by the peripheral nerves of the crab, Carcinus maenas (L). Biochim. Biophys. Acta 311:302-313, 1973.

12. Gerschenfeld, H. M. Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. Physiol. Rev. 53:1-119, 1973.

13. Gerschenfeld, H. and Tauc, L. Pharmacological specificities of neurones in an elementary central nervous system. Nature 189:924-925, 1961.

14. Giller, E., Jr. and Schwartz, J. H. Choline acetyltransferase in identified neurons of abdominal ganglion of Aplysia californica. J. Neurophysiol. 34:93-107, 1971.

15. Hattori, T., McGeer, P. L., Fibiger, H. C. and McGeer, E. G. On the source of GABA-containing terminals in the substantia nigra. Electron microscopic autoradiographic and biochemical studies. Brain Res. 54:103-114, 1973.

16. Henn, F. A., Goldstein, M. N. and Hamberger, A. Uptake of the neurotransmitter candidate glutamate by glia. Nature 249:663-664, 1974.

17. Henn, F. A. and Hamberger, A. Glial cell function: uptake of transmitter substances. Proc. Natl. Acad. Sci. USA 68:2686-2690, 1971.

18. Hökfelt, T. and Ljungdahl, A. Autoradiographic identification of cerebral and cerebellar cortical neurons accumulating labeled gamma-aminobutyric acid (^{3}H-GABA). Exp. Brain Res. 14:354-362, 1972.

19. Hutchison, H. T., Werrbach, K., Vance, C. and Haber, B. Uptake of neurotransmitters by clonal lines of astrocytoma and neuroblastoma in culture. I. Transport of γ-aminobutyric acid. Brain Res. 66:265-274, 1974.

20. Iversen, L. L. The uptake, storage, release, and metabolism of GABA in inhibitory nerves. In: Perspectives in Neuropharmacology, Snyder, S. H., editor, pp. 75-111. New York, Oxford University Press, 1972.

21. Iversen, L. L. and Kravitz, E. A. The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system -- uptake of GABA in nerve-muscle preparations. J. Neurochem. 15:609-620, 1968.

22. Lasek, R. J., Gainer, H. and Przybylski, R. J. Transfer of newly synthesized proteins from Schwann cells to the squid giant axon. Proc. Natl. Acad. Sci. USA 71:1188-1192, 1974.

23. Lasher, R. S. The uptake of [^{3}H]GABA and differentiation of stellate neurons in cultures of dissociated postnatal rat cerebellum. Brain Res. 69:235-254, 1974.

24. Ljungdahl, A. and Hökfelt, T. Autoradiographic uptake patterns of [^{3}H]GABA and [^{3}H]glycine in central nervous tissues with special reference to the cat spinal cord. Brain Res. 62:587-595, 1973.

25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

26. Martin, D. L. Kinetics of the sodium-dependent transport of gamma-aminobutyric acid by synaptosomes. J. Neurochem. 21:345-356, 1973.

27. Martin, D. L. and Smith, A. A., III. Ions and the transport of gamma-aminobutyric acid by synaptosomes. J. Neurochem. 19:841-855, 1972.

28. McCaman, R. E. and Dewhurst, S. A. Metabolism of putative transmitters in individual neurons of Aplysia californica. Acetylcholinesterase and catechol-O-methyl transferase. J. Neurochem. 18:1329-1335, 1971.

29. McCaman, R. E., Weinreich, D. and Borys, H. Endogenous levels of acetylcholine and choline in individual neurons of Aplysia. J. Neurochem. 21: 473-476, 1973.

30. Morin, W. A. and Atwood, H. L. A comparative study of gamma-aminobutyric acid uptake in crustacean nerve-muscle preparations. Comp. Biochem. Physiol. 30:577-583, 1969.

31. Orkand, P. M. and Kravitz, E. A. Localization of the sites of γ-aminobutyric acid (GABA) uptake in lobster nerve-muscle preparations. J. Cell. Biol. 49:75-89, 1971.

32. Osborne, N. N., Briel, G. and Neuhoff, V. Distribution of GABA and other amino acids in different tissues of the gastropod mollusc Helix pomatia, including in vitro experiments with ^{14}C glucose and ^{14}C glutamic acid. Int. J. Neurosci. 1:265-272, 1971.

33. Rose, S. P. R. and Cory, H. T. Glutamate metabolism in octopus brain in vivo; absence of a Waelsch effect. J. Neurochem. 17:817-820, 1970.

34. Schon, F. and Kelly, J. S. Autoradiographic localisation of [^{3}H]GABA and [^{3}H]glutamate over satellite glial cells. Brain Res. 66:275-288, 1974.

35. Schrier, B. K. and Thompson, E. J. On the role of glial cells in the mammalian nervous system. Uptake, excretion, and metabolism of putative neurotransmitters by cultured glial tumor cells. J. Biol. Chem. 249:1769-1780, 1974.

36. Seiler, N., Al-Therib, M. J. and Kataoka, K. Formation of GABA from putrescine in the brain of fish (Salmo irideus gibb). J. Neurochem. 20:699-708, 1973.

37. Seiler, N., Wiechmann, M., Fischer, H. A. and Werner, G. The incorporation of putrescine carbon into γ-aminobutyric acid in rat liver and brain in vivo. Brain Res. 28:317-325, 1971.

38. Sisken, B. and Roberts, E. Radioautographic studies of binding of γ-aminobutyric acid to the abdominal stretch receptors of the crayfish. Biochem. Pharmacol. 13:95-103, 1964.

39. Sotelo, C., Privat, A. and Drian, M. J. Localization of [^{3}H]GABA in tissue culture of rat cerebellum using electron microscopy radioautography. Brain Res. 45:302-308, 1972.

40. Trump, B. F., Smuckler, E. A. and Benditt, E. P. A method for staining epoxy sections for light microscopy. J. Ultrastruct. Res. 5:343-348, 1961.

41. Weinreich, D., McCaman, M. W., McCaman, R. E. and Vaughn, J. E. Chemical, enzymatic and ultrastructural characterization of 5-hydroxytryptamine-containing neurons from the ganglia of Aplysia californica and Tritonia diomedia. J. Neurochem. 20:969-976, 1973.

42. Young, J. A. C., Brown, D. A., Kelly, J. S. and Schon, F. Autoradiographic localization of sites of [^{3}H]γ-aminobutyric acid accumulation in peripheral autonomic ganglia. Brain Res. 63:479-486, 1973.

43. Zeman, G. H. and Carpenter, D. O. Putative neurotransmitters in Aplysia: distribution of gamma-aminobutyric acid, aspartate and glutamate in ganglia and single neurons. Bethesda, Maryland, Armed Forces Radiobiology Research Institute Scientific Report SR75-3, 1975.

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13. ABSTRACT

Selected ganglia of Aplysia dactylomela were found to possess a sodium dependent gamma-aminobutyric acid (GABA) uptake system. Tissue to medium ratios as high as 20:1 were calculated in the buccal ganglion after a 45-minute incubation in 6 μM GABA. The metabolism of accumulated GABA was low with 90 percent of the radioactivity remaining as GABA after a 45-minute incubation and 20-minute washout period. The buccal ganglion displayed a sodium dependent, high affinity uptake system with a K_m = 52 μM. No net sodium dependent accumulation was found in the pedal ganglion. In the buccal ganglion, light microscope autoradiography indicated that the sites of GABA accumulation were predominantly extraneuronal.

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